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Synthesis of rabdokunmin C analogues and their inhibitory effect on NF-κB activation

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ABSTRACT

A series of rabdokunmin C analogues were prepared and their inhibitory effect on NF-κB activation was assayed. One of them, 18-acetyl-12-deoxy-11,12-dehydrorabdokunmin C (**16**) was found to be a promising candidate for an anti-inflammatory agent.

1. Introduction

Nuclear factor kappa B (NF-κB), a principal inducible transcription factor in mammals, is known to play an important role in the mammalian immune response[1] and is also known to be highly activated in inflammation diseases such as rheumatoid arthritis, asthma, inflammatory bowel disease, and multiple sclerosis. Therefore, compounds having an inhibitory activity on NF-κB are considered to be candidates for anti-inflammatory drugs. Sodium salicylate and its derivative, aspirin,[2] and a number of natural products have been reported to have such an inhibitory effect on NF-κB activation.[3] As regards diterpenes, andalusol and a series of *ent*-kaurene diterpenoids are known to inhibit the NF-κB activation.[4–6] Lee et al. reported that natural 12-dehydroxy-19-acetylrabdokunmin C analogues from *Croton tonkinensis* inhibit LPS-induced NF-κB activation and NO production.[7] On the other hand, *ent*-kaurenes having a carboxyl or carboxaldehyde group at C-4 showed no or less cytotoxic against HepG2 cells.[8] That indicates that functional groups at C-4 may affect cytotoxicity. We previously reported isolation of natural *ent*-kaurene diterpenes from *Rabdosia excisa* and their cytotoxic activities on P388 murine leukemia cells,[9] with comments on the possible structure–activity relationship (SAR).[10] In the present study, we prepared a series of rabdokunmin C derivatives, having an *ent*-kaurene carbon skeleton as shown in Scheme 1 and assayed their inhibitory effects on the LPS-induced NF-kB activation and their possible structure–activity relationships.

2. Results and discussion

Rabdokunmin C analogues (**2**–**17**) were prepared as shown in Scheme 1. Thus, its acetonide **2** was obtained by the reaction of rabdokunmin C (**1**) with acetone in the presence of a catalytic amount of *p*-toluenesulfonic acid (PSA) in quantitative yield. Trifluoroacetylation and acetylation of **2** in the usual manner gave *12O,18O*-ditrifluoroacetylrabdokunmin C *7O,14O*-acetonide (**3**) and *12O,18O*-diacetylrabdokunmin C *7O,14O*-acetonide (**4**), respectively. Results of hydrolysis of **4** under different basic conditions are shown in Table 1. With 10% potassium carbonate, **4** gave complex products (entry 1), whereas with 10% sodium hydrogen carbonate for 8 h, it gave the monoacetate **5** in 55% yield (entry 2). To shorten the reaction time and to increase the yield, basic hydrolysis under irradiation with microwave was studied (entries 3–11). The highest yield of 64% of the aimed product **5** was obtained in entry 10, in which **4** was irradiated in 10% sodium bicarbonate by microwave, three times (10 min each) at 60 °C. The recovery of the starting material **4** was 26%. The structure of **5** was determined by the 1D and 2D NMR studies: The correlation between the carbonyl carbon of the acetyl group and the 18-methylene protons in HMBC spectrum confirmed that **5** is *18O*-acetylrabdokunmin C *7O,14O*-acetonide. Deacetalization of **5** gave a known

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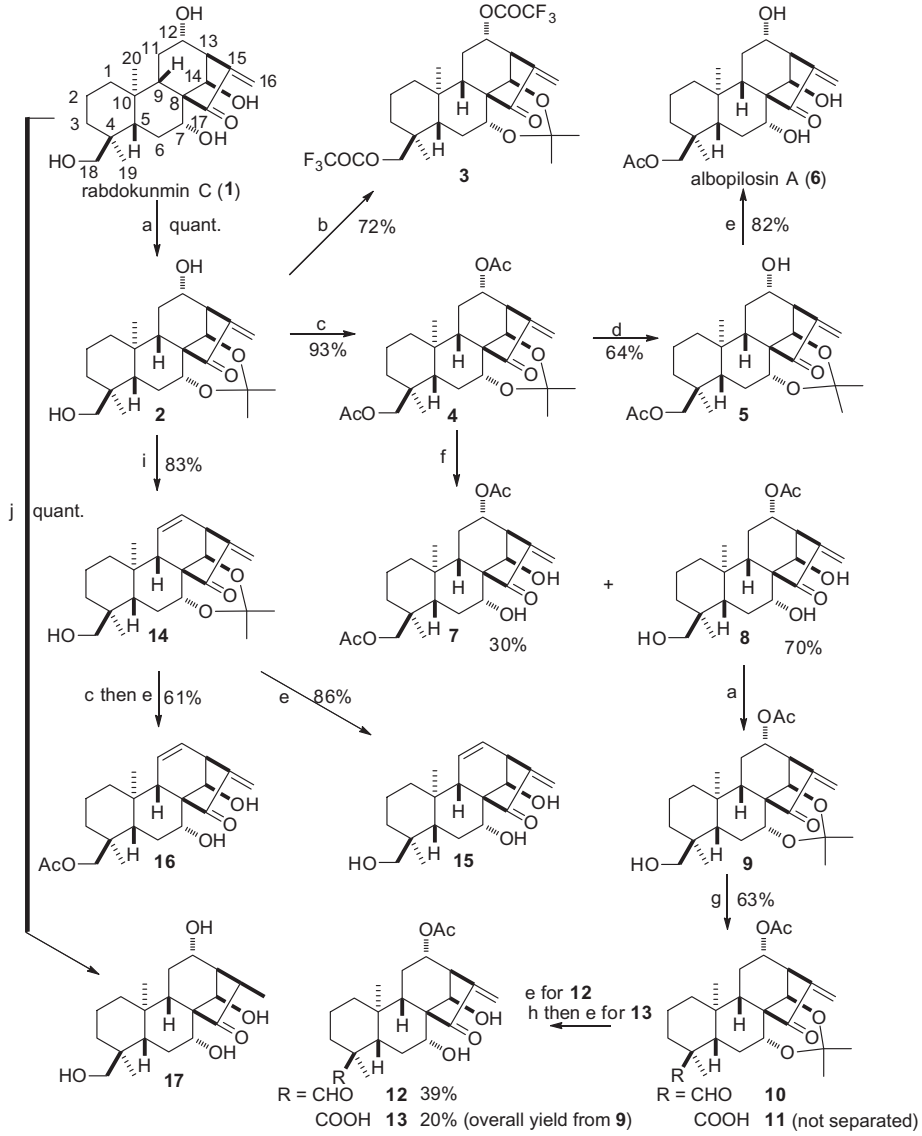


Scheme 1. Preparation of rabdokunmin C analogues. Reagents and conditions: (a) Me_2CO, PSA, rt; (b) $(CF_3CO)_2O$, pyridine, rt; (c) Ac_2O, pyridine, rt; (d) Refer to Table 1; (e) 1 N HCl, MeOH, rt; (f) Refer to Table 2; (g) Dess–Martin periodinane, CH_2Cl_2, rt; (h) $NaClO_2$, NaH_2PO_4, ^tBuOH, H_2O, 2-methyl-2-butene, rt; (i) PPh_3, DEAD, THF, rt; (j) 5% Pd–C/H_2/MeOH, rt.

Table 1
Microwave-assisted alkaline hydrolysis of **4** in MeOH–THF (1:5)

Entry	Conditions[a]	Yields[c] (%)		
		5	**2**	**4**
1[b]	10% K_2CO_3, rt, 2 h	—	—	—
2	10% $NaHCO_3$, 50 °C, 8 h	55	—	—
3	5% $NaHCO_3$, MW, 50 °C, 10 min × 2 times	35	—	50
4	5% $NaHCO_3$, MW, 80 °C, 10 min × 2 times	40	41	—
5	5% $NaHCO_3$, MW, 60 °C, 3 min × 2 times	33	—	56
6	10% $NaHCO_3$, MW, 80 °C, 3 min × 1 times	37	—	32
7	10% $NaHCO_3$, MW, 60 °C, 3 min × 2 times	20	—	64
8	10% $NaHCO_3$, MW, 60 °C, 10 min × 2 times	55	—	20
9	10% $NaHCO_3$, MW, 80 °C, 3 min × 2 times	33	—	34
10	10% $NaHCO_3$, MW, 60 °C, 10 min × 3 times	64	—	26
11	10% $NaHCO_3$, MW, 60 °C, 10 min × 4 times	57	—	14

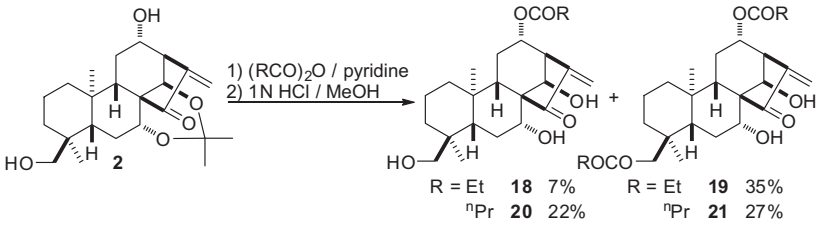
[a] MW: microwave irradiation.
[b] Complex products.
[c] Isolated yields.

compound, albopilosin A (**6**),[11] in 82% yield, whose spectral data were identical to those reported. Results of acid hydrolysis of **4** under several different reaction conditions are shown in Table 2. The aimed *12O*-acetylrabdokunmin C (**8**) was obtained in the best yield in entry 3, whose acetyl group was confirmed to be at C-12 by the correlation between the 12-methine proton and the carbonyl carbon of the acetyl group in HMBC spectrum. Then, **8** was reacetalized with acetone in the presence of a catalytic amount of PSA to give **9**, which was subjected to oxidation with Dess–Martin periodinane and sodium chlorite to give the corresponding carboxaldehyde **10** and acid **11**. Deacetalization of **10** and **11** under acidic

Table 2
Acidic hydrolysis of **4**

Entry	Conditions	Yields[a] (%)		
		7	**8**	**4**
1	1 N HCl–MeOH (1:5), rt, 15 h	45	—	42
2	1 N HCl–MeOH (1:10), rt, 15 h	62	26	—
3	1 N HCl–MeOH (1:10), rt, 48 h	30	70	—
4	1% I_2, MeOH, 50 °C, 2 h	28	43	—

[a] Isolated yields.



Scheme 2. Preparation of *18O*- and/or *12O*-mono and diacylrabdokunmin C analogues (**18**–**21**).

conditions gave **12** (39% yield) and **13** (20% overall yield from **10**), respectively. Dehydration of **2** under Mitsunobu reaction conditions gave **14** in 83% yield, which on hydrolysis, afforded **15** in 86% yield. On the other hand, when acetylated and then subjected to acidic hydrolysis, **14** gave **16** in 61% yield (overall from **14**). When hydrogenated, **1** gave **17** in a quantitative yield as reported.[12] *12O*-Mono- and *12O, 18O*-dipropionyl and valeroyl derivatives, **18**–**21** were prepared from **2** in the usual manner (Scheme 2).

3. Biological activity

As preliminary assay, the compounds of the present series, **1–9**, **12**–**17** and celastrol (**22**), a positive control triterpene,[13] were assayed for their inhibitory effect on the LPS-induced NO production by the RAW267 macrophage cells, at 5, 1, and 0.2 μg/mL. The results are shown in Figure 1 where the NO production is shown by the amount of NO detected and the viability as OD450 absorbance values. Thus, **4**–**9**, **12**, **15**, **16**, and **22** were shown to inhibit the NO production by the macrophage cells with weak cell killing effect, whereas **1**–**3**, **13**, **14** and **17** were less active with weaker cell killing effect. Among the radokunmin C series compounds, active ones, **4**–**9** and **12** have OAc groups at C-12 and/or C-18, whereas less active ones with weaker cell killing effect, **1**, **2**, and **17**, have OH groups both at C-12 and C-18. Accordingly, the one having a carboxyl group at C-18 was not active as reported for another analogue having a carboxyl group at C-18.[7] Among the $\Delta^{11,12}$-rabdokunmin C series compounds **14**, **15** and **16**, having no oxygen at C-12, **15**, having no acetoxy group was as active as its homologue **16**, having an acetoxy group at C-18, but **14**, an acetonide of **15**, was much less active. Perhaps a different factor such as conformational factor may more emphatically work in this series of compounds. The positive control **22** was shown to be very effective in inhibiting the NO production but at the same time it has a potential cell killing effect, suggesting the very low NO production was due to the death of the relevant cells.

Then, **4, 7, 9, 16,** and larger ester group-containing analogues **19**–**21,** were assayed for their inhibitory effect on the LPS-induced NF-κB activation and also on the viability of the cells along with positive controls, **22**, a known inhibitors of NF-κB activation[13] and oridonin (**23**), an *ent*-kaurene diterpenoid reported to be a strong inhibitor of NF-κB (Fig. 2).[14] The results are summarized in Table 3. The radokunmin C series compounds **4**, **7**, **9**, **20** and **21** all showed almost same inhibitory effect on the NF-κB activation and almost same cell killing effect, though **19**, having propionate groups both at C-12 and C-18 was less active. On the other hand, **16**, a $\Delta^{11,12}$-rabdokunmin C series compound, was shown to have an efficient inhibitory effect on the NF-κB cell activation with much weaker cell killing effect. Both of the positive controls **22** and **23**, especially **22**, showed efficient inhibitory effect on the NF-κB activation and also a potential cell killing, implying that the NF-κB cell activation–inhibition by them might be due to the killing of the cells.

Thus, the present assay showed that only **16** inhibited the LPS-induced NF-κB activation with little effect on the cell viability. Thus, **16** may be a possible candidate for an anti-inflammatory agent.

4. Experimental

4.1. General method

Melting points were determined on a Yanaco MP-3 apparatus and are recorded uncorrected. IR spectra were recorded on a JASCO FT/IR 620 spectrophotometer, optical rotation on a JASCO DIP-360 automatic digital polarimeter, and Mass spectra on a Micromass LCT (Manchester, UK) spectrometer. NMR spectra were recorded in $CDCl_3$ or pyridine-d_5 on a Brucker AM-400 and DRX-500 spectrometer at 300 K with the *J* values given in Hz. The chemical shifts (δ) are given in ppm relative to the residual $CHCl_3$ and pyridine-d_5 proton resonance at 7.26 and 7.23 ppm, respectively, for ^{1}H NMR, and to the $CDCl_3$ and pyridine-d_5 carbon resonance at 77.0 and 123.5 ppm, respectively, for ^{13}C NMR. Preparative HPLC was carried out on a JASCO PU-986 equipped with a UV-970 UV detector (λ 230 nm) and an Inertsil PREP-ODS column (10 μm, 20 × 250 mm), using a solvent system MeOH/H_2O or a MeCN/H_2O, at a flow rate of 10 mL/min.

4.2. Materials

Rabdokunmin C (**1**) was isolated from *R. excisa,* collected in Jing Yu county, Jinlin province of China, in August 2001.[9] Positive controls, **22** and **23** were purchased from EMD Chemicals, Inc. (NJ, USA) and Merck KGaA (Darmstadt, Germany), respectively.

4.3. Preparation of rabdokunmin C analogues

4.3.1. Preparation of 2

PSA (cat. ammount) was added to a solution of **1** (0.407 g, 1.16 mmol) in dry Me_2CO (40 mL) at rt under an Ar atmosphere. After stirring at rt for 3 h, satd $NaHCO_3$ (20 mL) was added to the mixture and its solvent was evaporated off in vacuo to leave an aqueous phase, which was extracted with AcOEt (20 mL × 3). The combined organic layer was washed with satd $NaHCO_3$ (30 mL × 3), dried over $MgSO_4$, and filtered. Its solvent was evaporated off in vacuo to give a brownish viscous oil. The oil was subjected to SiO_2-MPLC ($CHCl_3$/Me_2CO = 4:1) to give acetonide **2** as a colorless amorphous solid (0.45 g, quantitative yield). Its spectral and physical data were identical to those reported.[12]

4.3.2. Trifluoroacetylation of 2

A mixture of **2** (0.027 g, 0.069 mmol), $(CF_3CO)_2O$ (0.30 g, 0.2 mL, 1.43 mmol), and dry pyridine (0.98 g, 1.0 mL, 12.3 mmol) was stirred at 0 °C for 3.5 h and the reaction mixture was added to ice-water (20 mL). The aqueous phase was extracted with AcOEt (10 mL × 3) and the combined organic layer was washed with 1.7 N HCl (20 mL × 2), satd $NaHCO_3$ (20 mL × 2), and satd NaCl

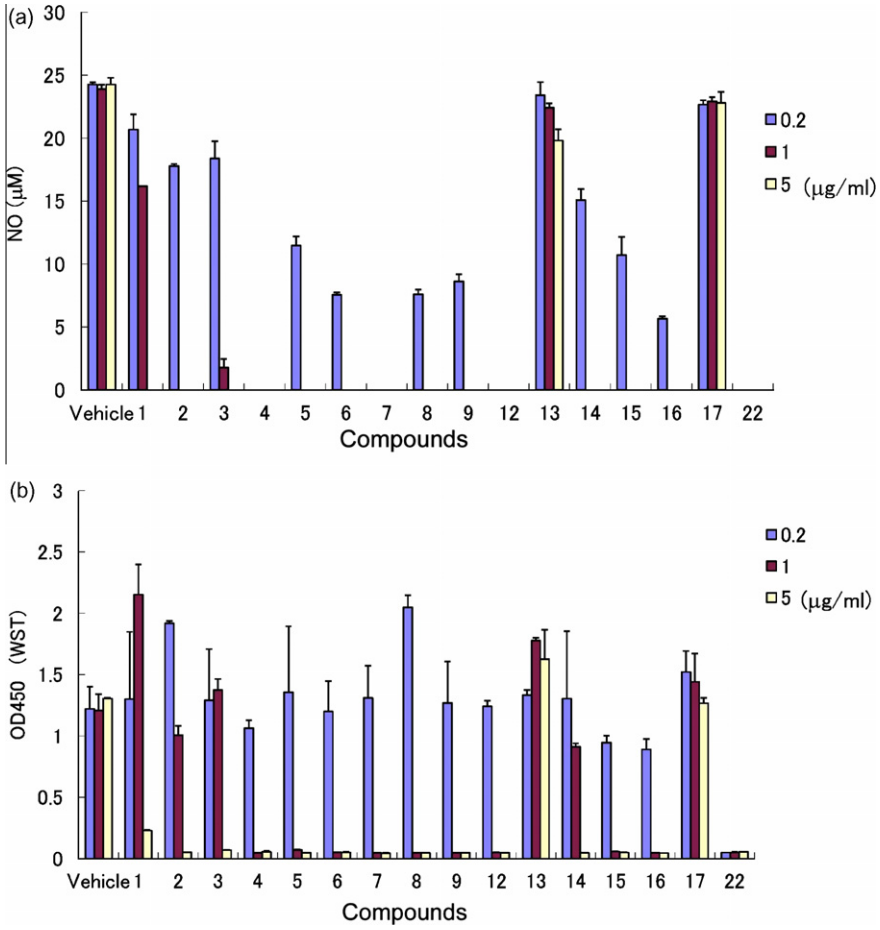


Figure 1. Effect of compounds **1**–**9**, **12**–**17**, and **22** on LPS-induced NO production by macrophages and on cell viability expressed as OD450.

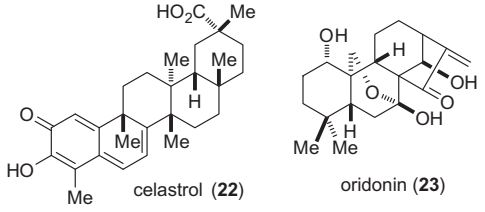


Figure 2. Positive controls.

Table 3
IC_{50} on NF-κB activation and cell viability

Compd	IC_{50} (μM)			
	CV[b]	SD[d]	NF-κB[c]	SD[d]
4	1.80	0.07	2.01	0.09
7	2.92	0.41	2.00	0.08
9	2.39	0.27	1.53	0.11
16	>5.00		1.47	0.14
19	>5.00		>5.00	
20	1.91	0.15	1.68	0.08
21	2.30	0.19	2.97	0.80
22[a]	0.67	0.04	0.17	0.02
23[a]	>5.00		4.44	0.43

[a] Positive control: **22**: celastrol. **23**: oridonin.
[b] Viability of 293T cells transfected with NF-κB luciferase reporter gene.
[c] Inhibitory effect on LPS-induced NF-κB activation.
[d] Standard deviation.

(20 mL × 2), successively, dried over $MgSO_4$, and filtered. The solvent was evaporated off in vacuo to give an oily residue, which was subjected to SiO_2-MPLC (hexanes/AcOEt = 6:1) to give **3** (0.029 g, 72%) as a colorless amorphous solid. Mp 66–68 °C ($CHCl_3$). $[\alpha]_D$ −37.4 (*c* 0.42, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ: 6.29 (1H, s), 5.59 (1H, s), 5.28 (1H, dd, 4.5, 4.5), 4.79 (1H, d, 0.9), 4.25 (1H, dd, 12.3, 6.1), 4.09 (1H, d, 10.9), 4.02 (1H, d, 10.9), 3.28 (1H, d, 3.6), 1.99 (1H, ddd, 12.6, 12.6, 12.6), 1.94–1.88 (2H, m), 1.81 (1H, d, 17.2), 1.64–1.45 (5H, m), 1.58 (3H, s), 1.28 (1H, m), 1.23 (3H, s), 1.14 (3H, s), 1.11 (1H, dd, 12.2, 2.2), 0.96 (3H, s), 0.74 (1H, ddd, 12.2, 12.2, 4.0). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 204.2, 157.4, 156.5, 142.0, 120.6, 114.5, 114.4, 97.7, 77.7, 75.5, 69.9, 66.1, 53.6, 51.6, 46.8, 45.3, 38.8, 37.7, 36.7, 35.2, 30.5, 27.3, 25.0, 22.7, 17.13, 17.10, 16.0. IR (film): ν_{max} 1786, 1741 (C=O), 1653 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{27}H_{33}F_6O_7$ (M^++H): 583.2130. Found: 583.2124.

4.3.3. Acetylation of 2

A mixture of **2** (0.32 g, 0.83 mmol), dry pyridine (3 mL, 2.93 g, 37.1 mmol), and Ac_2O (1.5 mL, 1.62 g, 15.9 mmol) was stirred at rt for 15 h. The mixture was poured into ice-water (20 mL) and the pH was adjusted to 8.0 with powdered $NaHCO_3$. The aqueous solution was extracted with $CHCl_3$ (10 mL × 3). The organic phase was separated, washed with satd NaCl (10 mL × 3), dried over $MgSO_4$, filtered, and then subjected to evaporation in vacuo to give an oily residue. The residue was subjected to SiO_2-MPLC (hexane/AcOEt = 2:1) to give **4** (0.38 g, 96%) as a **c**olorless amorphous solid. Mp 173–175 °C. $[\alpha]_D^{25}$ −46 (*c* 0.26, $CHCl_3$). ^1H NMR (400 MHz, 300 K, $CDCl_3$) δ 6.23 (1H, s), 5.53 (1H, s), 5.09 (1H, m), 4.81 (1H, d, *J* = 1.1),

4.25 (1H, m), 3.84 (1H, d, J = 11.2), 3.69 (1H, d, J = 11.2), 3.17 (1H, d, J = 3.5), 2.13 (3H, s), 2.08 (3H, s), 1.94 (1H, m), 1.92 (1H, m), 1.79 (1H, m), 1.70 (1H, m), 1.62–1.43 (3H, m, overlapped), 1.59 (3H, s), 1.36 (2H, m, overlapped), 1.26–1.13 (2H, m, overlapped), 1.23 (3H, s), 1.15 (3H, s), 0.88 (3H, s), 0.71 (1H, ddd, J = 12.3, 12.3, 4.0). ^{13}C NMR (100 MHz, 300 K, $CDCl_3$) δ 205.5, 171.0, 169.8, 143.2, 119.4, 97.4, 73.2, 71.9, 70.2, 66.6, 53.9, 52.2, 47.4, 44.8, 38.8, 37.6, 36.2, 35.4, 30.6, 27.1, 25.2, 23.0, 21.3, 20.9, 17.5, 17.3, 16.2. IR (film) 1739 (C=O), 1653 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{27}H_{39}O_7$: 475.2696 (M^++H). Found: 475.2673.

4.3.4. Microwave-assisted alkaline hydrolysis of 4

A mixture of **4** (0.005 g, 0.0105 mmol), 10% $NaHCO_3$ (0.025 mL), MeOH (0.25 mL), and THF (0.05 mL) was irradiated in a sealed glass tube at 60 °C by using a microwave apparatus (at 2.45 GHz, Discover, CEM Co., North Carolina, USA) (10 min × 3). After dilution with H_2O (10 mL), the mixture was extracted with AcOEt (10 mL × 3), and the extract was washed with satd NaCl (20 mL × 2), dried over $MgSO_4$, filtered, and subjected to evaporation in vacuo to give an oily residue. The residue was subjected to PTLC ($CHCl_3$/Me_2CO = 7:1) to give **5** (0.029 g, 64%) as colorless prisms and the recovered starting material **4** (0.0013 g, 26%).

Compound **5**: Mp 216–218 °C (MeOH). $[\alpha]_D$ −65.8 (c 0.90, $CHCl_3$). ^{1}H NMR (600 MHz, 300 K, $CDCl_3$) δ 6.16 (1H, s), 5.40 (1H, s), 4.92 (1H, s), 4.24 (1H, 11.6, 6.8), 4.17 (1H, dd, 4.3, 4.3), 3.83 (1H, d, 11.2), 3.68 (1H, d, 11.2), 3.07 (1H, d, 3.3), 2.07 (3H, s), 1.99 (1H, br s), 1.93 (1H, dd, 12.2, 12.2), 1.92 (1H, m, overlapped), 1.71 (1H, ddd, 16.7, 9.6, 5.3), 1.67 (1H, m), 1.65 (1H, m, overlapped), 1.58 (3H, s), 1.58 (1H, m, overlapped), 1.47 (1H, m), 1.42 (1H, d, 9.2), 1.34 (1H, m, overlapped), 1.34 (1H, dd, 9.8, 3.4), 1.24 (3H, s), 1.23 (3H, s), 1.18 (1H, dd, 11.5, 3.2), 0.87 (3H, s), 0.70 (1H, ddd, 13.1, 13.1, 3.5). ^{13}C NMR (150 MHz, 300 K, $CDCl_3$) δ 206.5, 171.2, 144.2, 118.1, 97.4, 72.0, 70.3, 66.3, 54.3, 53.2, 51.0, 44.9, 38.8, 37.5, 36.3, 35.5, 30.7, 27.1, 25.6, 25.2, 21.0, 17.5, 17.4, 16.4. IR (film) 3437 (OH), 1736 (C=O), 1645 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{25}H_{37}O_6$: 433.2590 (M^++H). Found: 433.2552.

4.3.5. Preparation of albopilosin A (6)

A mixture of **5** (0.0077 g, 0.0198 mmol), 1 N HCl (0.2 mL), and MeOH (1.0 mL) was stirred at rt for 4 h. After dilution with H_2O (10 mL), the mixture was extracted with AcOEt (10 mL × 3), and the combined extract was washed with satd $NaHCO_3$ (20 mL × 2) and satd NaCl (20 mL × 2), dried over $MgSO_4$, and filtered. The filtrate was subjected to evaporation in vacuo to give colorless amorphous solid, which was subjected to HPLC (60% MeCN, v/v) to give **6** (0.0057 g, 82%) as a colorless solid (MeOH–H_2O), whose physical data were identical with those reported.[11]

4.3.6. Acidic hydrolysis of 4

A mixture of **4** (0.010 g, 0.021 mmol), 1 N HCl (0.1 mL), and MeOH (1.0 mL) was stirred at rt for 48 h. After dilution with H_2O (10 mL), the mixture was extracted with AcOEt (10 mL × 3), and the combined extract was washed with satd $NaHCO_3$ (20 mL × 2) and satd NaCl (20 mL × 2), dried over $MgSO_4$, filtered, and subjected to evaporation in vacuo to give colorless amorphous solid. PTLC (hexane/Me_2CO/$CHCl_3$ = 10:5:4) of the solid gave **7** (0.0028 g, 30%) as a colorless solid and **8** (0.0058 g, 70%) as a colorless solid.

Compound **7**: Colorless amorphous solid, mp 235–237 °C ($CHCl_3$). $[\alpha]_D$ −46.3 (c 0.16, $CHCl_3$). ^{1}H NMR (400 MHz, 300 K, $CDCl_3$) δ 6.26 (1H, s), 5.57 (1H, s), 5.39 (1H, br s), 5.11 (1H, s), 5.02 (1H, J = 7.1, 3.7), 4.41 (1H, m), 3.91 (1H, d, J = 11.1), 3.66 (1H, d, J = 11.1), 3.19 (1H, d, J = 3.1), 2.97 (1H, br s), 2.104 (3H, s), 2.095 (3H, s), 1.87 (1H, m), 1.78 (1H, ddd, J = 12.0, 12.0, 12.0), 1.71 (2H, m, overlapped), 1.65–1.57 (2H, m, overlapped), 1.52–1.47 (1H, m), 1.42 (1H, dd, J = 5.3, 5.3), 1.37–1.34 (2H, m, overlapped), 1.25 (1H, m), 1.21 (3H, s), 0.89 (3H, s), 0.69 (1H, m). ^{13}C NMR (100 MHz, 300 K, $CDCl_3$) δ 206.3, 170.3, 168.9, 142.9, 119.7, 73.7, 72.4, 71.2, 69.6, 59.8, 53.4, 49.2, 45.6, 37.7, 37.5, 35.4, 34.2, 26.9, 22.0, 20.4, 20.1, 16.6, 16.5, 15.2. IR (film) 3383 (OH), 1734 (C=O), 1646 (C=C). HRMS (ESI): Calcd for $C_{24}H_{34}O_7Na$: 457.2202 (M^++Na). Found: 457.2210.

Compound **8**: Colorless prisms, mp 226–228 °C (MeOH). $[\alpha]_D$ −54.2 (c 0.29, MeOH). ^{1}H NMR (400 MHz, 300 K, pyridine-d_5) δ 8.24 (1H, d, 5.1), 7.40 (1H, s), 6.33 (1H, s), 6.16 (1H, dd, 5.2, 5.2), 5.53 (1H, s), 5.48 (1H, s), 5.21 (1H, dd, 4.6, 4.6), 5.04 (1H, ddd, 12.1, 4.6, 4.6), 3.68 (1H, dd, 10.6, 5.2), 3.54 (1H, d, 3.4), 3.34 (1H, 10.6, 5.2), 2.45 (1H, m), 2.15 (3H, s), 2.05 (1H, ddd, 12.1, 12.1, 12.1), 1.85–1.58 (6H, m, overlapped), 1.51 (1H, m), 1.44–1.36 (2H, m, overlapped), 1.29 (3H, s), 0.91 (3H, s), 0.62 (1H, m). ^{13}C NMR (100 MHz, 300 K, pyridine-d_5) δ 207.7, 170.0, 146.1, 118.8, 74.4, 74.3, 71.239, 71.199, 61.1, 55.4, 51.2, 46.2, 39.4, 38.7, 37.9, 35.5, 29.5, 23.31, 21.27, 18.3, 18.1, 16.5. IR (film) 3346 (OH), 1732 (C=O), 1645 (C=C). HRMS (ESI): Calcd for $C_{22}H_{33}O_6$: 393.2277 (M^++H). Found: 393.2287.

4.3.7. Preparation of 9

A mixture of **8** (0.0082 g, 0.021 mmol) and a catalytic amount of PSA in dry Me_2CO (5 mL) was stirred at rt for 6 h. After addition of satd $NaHCO_3$ (20 mL), the mixture was subjected to evaporation in vacuo to leave an aqueous phase, which was extraced with AcOEt (20 mL × 3). The combined organic layer was washed with satd $NaHCO_3$ (30 mL × 3), dried over $MgSO_4$, filtered, and subjected to evaporation in vacuo to give a brownish viscous oil. HPLC (50% MeCN, v/v) of the oil gave acetonide **9** as a colorless amorphous solid (0.054 g, 60%). Mp 197–198 °C (MeOH–H_2O). $[\alpha]_D$ −53.5 (c 0.20, $CHCl_3$). ^{1}H NMR (400 MHz, 300 K, $CDCl_3$) δ 6.22 (1H, s), 5.52 (1H, s), 5.09 (1H, m), 4.81 (1H, d, 1.3), 4.29 (1H, dd, 11.8, 6.5), 3.42 (1H, d, 10.8), 3.152 (1H, d, 10.8), 3.154 (1H, d, m, overlapped), 2.13 (3H, s), 1.94 (1H, m), 1.90 (1H, ddd, 12.3, 12.3, 12.3), 1.79 (1H, m), 1.70 (1H, d, 17.1), 1.594 (3H, s), 1.593 (1H, m, overlapped), 1.57 (1H, m, overlapped), 1.51 (1H, m, overlapped), 1.43 (1H, m, overlapped), 1.40 (1H, m, overlapped), 1.31 (1H, m), 1.23 (3H, s), 1.21 (1H, m, overlapped), 1.15 (3H, s), 0.82 (3H, s), 0.70 (1H, m). ^{13}C-NMR (100 MHz, 300 K, $CDCl_3$) δ 205.7, 169.9, 143.3, 119.5, 97.4, 73.3, 71.6, 70.3, 66.7, 54.0, 52.3, 47.5, 44.4, 39.0, 37.6, 37.4, 34.9, 30.6, 27.1, 25.2, 23.0, 21.4, 17.5, 17.4, 16.3. IR (film) 3383 (OH), 1738 (C=O), 1649 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{25}H_{37}O_6$: 433.2590 (M^++H). Found: 433.2587.

4.3.8. Preparation of 10

Dess–Martin periodinane (0.0128 g, 0.03 mmol) was added to a solution of **9** (0.0067 g, 0.0155 mmol) in CH_2Cl_2 (1 mL) at 0 °C. After stirring at room temperature for 1 h, satd $Na_2S_2O_3$ (5 mL) and satd $NaHCO_3$ (5 mL) were added to the reaction mixture. The resulting mixture was stirred at rt for 0.5 h, and then extracted with AcOEt (10 mL × 3). The combined organic layer was washed with satd NaCl (20 mL × 2), dried over $MgSO_4$, and filtered and the solvent was evaporated in vacuo to give an oily residue, which, by MPLC (hexane/AcOEt = 2:1) gave **10** as a colorless solid (0.0069 g, 63%). Mp 166–168 °C (MeOH–H_2O). $[\alpha]_D$ −51.3 (c 0.20, $CHCl_3$). ^{1}H NMR (400 MHz, 300 K, $CDCl_3$) δ 9.22 (1H, s), 6.24 (1H, s), 5.54 (1H, s), 5.09 (1H, dd, 4.3, 4.3), 4.80 (1H, d, 0.96), 4.31 (1H, dd, 12.6, 5.9), 3.17 (1H, d, 3.5), 2.13 (3H, s), 2.01 (1H, ddd, 12.8, 12.8, 12.8), 1.82 (1H, ddd, 17.2, 10.0, 5.9), 1.70 (1H, d, 16.5), 1.65–1.47 (6H, m), 1.43–1.24 (3H, m), 1.56 (3H, s), 1.21 (3H, s), 1.17 (3H, s), 1.11 (3H, s), 0.78 (1H, m). ^{13}C NMR (100 MHz, 300 K, $CDCl_3$) δ 205.2, 205.1, 169.9, 143.0, 119.8, 97.5, 73.1, 69.7, 66.6, 54.0, 52.1, 49.3, 47.4, 43.5, 38.5, 36.6, 32.1, 30.6, 29.5, 25.1, 22.9, 21.4, 16.7, 16.1, 14.1. IR (film) 1737 (C=O), 1651 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{25}H_{35}O_6$: 431.4234 (M^++H). Found: 431.2412.

4.3.9. Preparation of 12

A mixture of **10** (0.0057 g, 0.0132 mmol), 1 N HCl (0.2 mL), and MeOH (1.0 mL) was stirred, after stirring at rt for 8 h, diluted with H_2O (10 mL), and extracted with AcOEt (10 mL × 3). The combined organic layer was washed with satd NaCl (20 mL × 2), dried over $MgSO_4$, filtered, and subjected to evaporation in vacuo to give colorless amorphous solid. HPLC (60% MeOH, v/v) of the crude solid gave **12** (0.002 g, 39%) as a colorless solid. Mp 220–222 °C (MeOH–H_2O). $[\alpha]_D$ −42.3 (*c* 0.094, MeOH). ^{1}H NMR (600 MHz, 300 K, pyridine-d_5) δ 9.28 (1H, s), 8.36 (1H, d, 5.3), 7.32 (1H, s), 6.36 (1H, s), 5.51 (1H, s), 5.45 (1H, s), 5.21 (1H, br s), 4.94 (1H, m), 3.54 (1H, d, 3.3), 2.15 (3H, s), 2.10 (1H, ddd, 12.7, 12.7, 12.7), 1.76–1.72 (3H, m, overlapped), 1.61–1.46 (4H, m, overlapped), 1.38 (1H, m), 1.29 (1H, ddd, 13.1, 13.1, 4.3), 1.20 (3H, s), 1.15 (1H, m, overlapped), 1.14 (3H, s), 0.58 (1H, ddd, 13.0, 13.0, 3.2). ^{13}C NMR (150 MHz, 300 K, pyridine-d_5) δ 207.2, 205.8, 170.0, 145.8, 119.3, 74.1, 73.4, 71.1, 61.0, 54.6, 51.1, 49.6, 44.9, 38.3, 37.6, 32.1, 32.0, 23.0, 21.2, 17.1, 16.1, 14.2. IR (film) 3377 (OH), 1736, 1711 (C=O), 1649 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{22}H_{31}O_6$: 391.2121 (M^++H). Found: 391.2143.

4.3.10. Preparation of 13

To a solution of **10** (0.014 g, 0.0325 mmol) in ^{t}BuOH 0.6 mL), 2-methyl-2-butene (0.43 mL, 0.287 g, 4.1 mmol) and a solution of $NaClO_2$ (0103 g, 0.911 mmol) and NaH_2PO_4 (0.058 g, 0.488 mmol) were added, successively. The reaction mixture was stirred at rt for 0.5 h. After addition of H_2O (5 mL), the aqueous phase was extracted with AcOEt (10 mL × 3). The combined organic layer was washed with H_2O (15 mL), 10% citric acid (15 mL), and satd NaCl (15 mL), successively. The combined organic extract was dried over $MgSO_4$, and filtered, and the solvent was evaporated off in vacuo to give a brownish viscous oil. The oil was subjected to MPLC (hexane/AcOEt = 1:1) to give a crude acid **11** as an amorphous solid, which (0.0063 g, 0.0135 mmol), without further purification, was mixed with 1 N HCl (0.2 mL), and MeOH (1.0 mL), and stirred at rt for 15 h. After dilution with H_2O (10 mL), the mixture was extracted with AcOEt (10 mL × 3), and the extract was washed with satd NaCl (20 mL × 2), dried over $MgSO_4$, and filtered, and the solvent was evaporated off in vacuo to give colorless amorphous solid, which, by HPLC (57% MeCN, v/v), gave **13** (0.0026 g, 20%, overall yield) as a colorless solid. Mp 253–255 °C (dec.) (MeOH–H_2O). $[\alpha]_D$ −41.1 (*c* 0.129, MeOH). ^{1}H NMR (600 MHz, 300 K, CD_3OD) δ 6.18 (1H, s), 5.56 (1H, s), 5.10 (1H, s), 4.98 (1H, dd, 4.7, 4.7), 4.33 (1H, m), 3.13 (1H, d, 3.5), 2.11 (3H, s), 1.83–1.52 (10H, m, overlapped), 1.46 (1H, d, 9.2), 1.25 (3H, s), 1.22 (3H, s), 0.83 (1H, m). ^{13}C NMR (150 MHz, 300 K, CD_3OD) δ 207.9, 182.8, 171.7, 146.0, 120.3, 75.4, 74.9, 72.1, 61.5, 56.7, 51.8, 48.4, 48.3, 39.9, 39.2, 37.9, 32.6, 23.7, 21.3, 18.8, 17.2, 16.7. IR (film) 3348 (OH), 1730 (C=O), 1650 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{22}H_{31}O_7$: 407.2070 (M^++H). Found: 407.2069.

4.3.11. Preparation of 14

To a solution of **2** (0.02 g, 0.0512 mmol), PPh_3 (0.040 g, 0.154 mmol), and benzoic acid (0.016 g, 0.128 mmol) in dry THF (2 mL), diethyl azodicarboxylate (0.023 mL, 0.022 g, 0.128 mmol) was added at 0 °C under an Ar atmosphere. The mixture was stirred at rt overnight. The solvent was evaporated in vacuo to give an oily residue, which was subjected to MPLC (hexanes/AcOEt = 2:1) to give **14** (0.0159 g, 83%) as a colorless solid. Mp 93–95 °C ($CHCl_3$). $[\alpha]_D$ −238.3 (*c* 0.42, $CHCl_3$). ^{1}H NMR (500 MHz, 300 K, $CDCl_3$) δ 6.04 (1H, ddd, 9.4, 7.1, 2.0), 5.86 (1H, s), 5.49 (1H, dd, 9.7, 3.2), 5.16 (1H, s), 4.67 (1H, d, 1.9), 4.37 (1H, dd, 12.5, 6.3), 3.43 (1H, d, 10.8), 3.30 (1H, d, 7.0), 3.18 (1H, d, 10.8), 1.97 (1H, ddd, 13.0, 6.3, 2.1), 1.88 (1H, ddd, 12.5, 12.5, 12.5), 1.87 (1H, m, overlapped), 1.72 (1H, br d), 1.59 (3H, s), 1.58 (1H, m, overalpped), 1.53 (1H, m), 1.43 (1H, ddd, 13.1, 13.1, 4.2), 1.34 (1H, m), 1.29 (1H, dd, 12.3, 2.1), 1.25 (3H, s), 1.03 (3H, s), 0.95 (1H, 12.8, 12.8, 4.2), 0.82 (3H, s). ^{13}C NMR (125 MHz, 300 K, $CDCl_3$) δ 207.3, 147.5, 130.8, 125.9, 112.4, 97.5, 71.7, 70.4, 68.8, 56.1, 52.4, 44.3, 43.8, 38.8, 37.8, 37.5, 34.9, 30.6, 27.3, 25.2, 17.8, 17.6, 17.5. IR (film) 3454 (OH), 1734 (C=O), 1651 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{23}H_{33}O_4$: 373.2379 (M^++H). Found: 373.2384.

4.3.12. Preparation of 15

A mixture of **14** (0.0046 g, 0.012 mmol), 1 N HCl (0.1 mL), and MeOH (1.0 mL) was stirred at rt for 40 h. After dilution with H_2O (10 mL), the mixture was extracted with AcOEt (10 mL × 3), and the combined organic layer was washed with satd $NaHCO_3$ (20 mL × 2) and NaCl (20 mL × 2), dried over $MgSO_4$, and filtered. The solvent was removed by evaporation in vacuo to give a colorless solid, which was subjected to HPLC (30% MeCN, v/v) to give **15** (0.0032 g, 86%) as a colorless solid. Mp 138–140 °C (MeOH). $[\alpha]_D$ −54.2 (*c* 0.05, $CHCl_3$). ^{1}H NMR (600 MHz, 300 K, pyridine-d_5) δ 8.17 (1H, br d), 7.54 (1H, br s), 6.19 (1H, br t), 6.04 (1H, dd 7.4, 7.4), 6.00 (1H, s), 5.42 (1H, m), 5.35 (1H, s), 5.19 (1H, s), 5.08 (1H, m), 3.67 (1H, m), 3.54 (1H, d, 6.6), 3.33 (1H, m), 2.43 (1H, d, 12.5), 2.06 (1H, s), 2.00 (1H, ddd, 12.5, 12.5, 12.5), 1.87 (1H, d, 12.5), 1.81 (1H, dd, 13.1, 13.1),1.60 (1H, m), 1.56 (1H, d, 13.1), 1.42 (1H, m), 1.37 (1H, d, 12.6), 1.08 (3H, s), 0.86 (3H, s), 0.86 (1H, m, overlapped). ^{13}C NMR (150 MHz, 300 K, pyridine-d_5) δ 209.3, 150.4, 132.4, 126.1, 111.5, 74.5, 73.5, 71.1, 59.9, 58.9, 48.1, 45.5, 39.0, 38.9, 37.9, 35.4, 29.4, 18.3, 18.1, 18.0. IR (film) 3442 (OH), 1726 (C=O), 1643 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{20}H_{29}O_4$: 333.2066 (M^++H). Found: 333.2040.

4.3.13. Preparation of 16

A mixture of **14** (0.014 g, 0.0376 mmol), dry pyridine (0.5 mL, 0.49 g, 6.2 mmol), and Ac_2O (0.2 mL, 0.216 g, 2.12 mmol) was stirred at room temperature for 15 h. The mixture was poured into ice-water (10 mL), which was extracted with AcOEt (10 mL × 3). The combined organic layer was then washed with 5% HCl (10 mL × 3), satd $NaHCO_3$ (10 mL × 3), and satd NaCl (10 mL × 3), dried over $MgSO_4$, and filtered. The solvents was evaporated in vacuo to give an oily residue (0.0134 g), which was subjected to acidic hydrolysis without further purification, by stirring with 1 N HCl (0.1 mL) and MeOH (1.0 mL) at rt for 3.5 h. After dilution with H_2O (10 mL), the hydrolysate mixture was extracted with AcOEt (10 mL × 3), and the extract was washed with satd $NaHCO_3$ (10 mL × 3) and satd NaCl (10 mL × 3), successively. The organic layer was dried over $MgSO_4$, and filtered. The filtrate gave, after evaporation of the solvent in vacuo, colorless viscous oil, which was subjected to HPLC (45% MeCN, v/v) to give **16** (0.0071 g, 61%, overall yield from **14**) as a colorless solid. Mp 168–169 °C (MeOH–H_2O). $[\alpha]_D$ −252.2 (*c* 0.14, MeOH). ^{1}H NMR (600 MHz, 300 K, pyridine-d_5) δ 8.11 (1H, d, 4.4), 7.32 (1H, s), 8.26 (1H, d, 5.3), 7.45 (1H, s), 6.05 (1H, d, 7.0), 6.03 (1H, s), 5.40 (1H, dd, 9.5, 3.5), 5.30 (1H, s), 5.20 (1H, s), 4.89 (1H, m), 4.05 (1H, d, 11.0), 3.73 (1H, d, 11.0), 3.54 (1H, d, 6.9), 2.14 (1H, dd, 13.0, 4.3), 2.02 (1H, br s), 1.96 (1H, ddd, 12.5, 12.5, 12.5), 1.88 (3H, s), 1.524 (1, d, 9.1), 1.515 (1H, m), 1.45 (1H, d, 12.1), 1.38 (1H, m), 1.37 (1H, d, 10.7), 1.30 (1H, d, 14.0), 1.01 (3H, s), 0.80 (3H, s), 0.79 (1H, m, overlapped). ^{13}C NMR (150 MHz, 300 K, pyridine-d_5) δ 208.9, 170.7, 209.1, 170.6, 132.7, 125.8, 111.8, 74.2, 73.3, 72.5, 59.8, 58.6, 48.1, 46.3, 38.9, 38.7, 36.5, 35.5, 29.6, 20.5, 17.91, 17.88, 17.6. IR (film) 3346 (OH), 1730, 1718 (C=O), 1645 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{22}H_{31}O_5$: 375.2171 (M^++H). Found: 375.2164.

4.3.14. Preparation of 17

Hydrogenation of **1** (0.01 g, 0.029 mmol) was performed in MeOH (1.0 mL) over 5% Pd–C (5 mg) under a hydrogen atmosphere. The catalyst was filtered off through Celite 545 and the

residue was washed with MeOH. The filtrate and washings were evaporated in vacuo to give a visucous oil, which was subjected to HPLC (40% MeOH, v/v) to give **17** (10 mg, quantitative yield) as an amorphous solid. The spectral data of the product were identical to those reported.[12]

4.3.15. Preparation of 18 and 19 from 2

A mixture of **2** (0.033 g, 0.085 mmol), propionyl anhydride (0.033 g, 0.033 mL, 0.255 mmol), dry Et_3N (0.034 g, 0.047 mL, 0.34 mmol), a catalytic amount of DMAP, and dry CH_2Cl_2 (1 mL) was stirred at rt for 3 h. The reaction mixture was diluted with AcOEt (20 mL). The aqueous layer was extracted with AcOEt (10 mL × 3) and the combined AcOEt layer was washed with 5% HCl (20 mL × 2), satd $NaHCO_3$ (20 mL × 2), and satd NaCl (20 mL × 2), successively, and dried over $MgSO_4$, and filtered. The solvent was evaporated in vacuo to give an oily residue, which was subjected to SiO_2-MPLC (hexane/AcOEt = 4:1) to give a crude diacyl compound (0.031 g). A mixture of the crude diacyl compound (0.020 g, 0.040 mmol), 1 N HCl (0.8 mL), and MeOH (4.0 mL) was stirred at rt for 48 h. After dilution with H_2O (30 mL), the mixture was extracted with AcOEt (15 mL × 3), and the extract was washed with satd $NaHCO_3$ (20 mL × 2) and NaCl (20 mL × 2), successively, dried over $MgSO_4$, and filtered. The solvent was evaporated in vacuo to give a colorless solid, which, by HPLC (75% MeOH, v/v), gave **18** (0.016 g, 7%, overall yield) and **19** (0.088 g, 35%, two steps overall yield), both as colorless solid.

Compound **18**: Mp 203–205 °C (MeOH). $[\alpha]_D$ −45.8 (*c* 0.09, MeOH). ^{1}H NMR (500 MHz, 300 K, pyridine-d_5) δ 8.26 (1H, d, 5.0), 7.43 (1H, s), 6.34 (1H, s), 6.18 (1H, dd, 5.0, 5.0), 5.54 (1H, s), 5.49 (1H, s), 5.24 (1H, dd, 4.9, 4.9), 5.05 (1H, ddd, 12.1, 4.5, 4.5), 3.69 (1H, dd, 10.6, 5.0), 3.61 (1H, d, 4.4), 3.34 (1H, dd, 10.6, 5.0), 2.74–2.43 (2H, m), 2.07 (1H, ddd, 12.4, 12.4, 12.4), 1.84–1.63 (5H, m), 1.52 (1H, br d), 1.43–1.28 (4H, m), 1.32 (3H, s), 1.17 (3H, t, 7.7), 0.92 (3H, s), 0.62 (1H, m). ^{13}C NMR (125 MHz, 300 K, pyridine-d_5) δ 207.7, 173.3, 146.2, 118.8, 74.32, 74.30, 71.3, 71.2, 61.1, 55.4, 51.3, 46.2, 39.4, 38.8, 38.0, 35.5, 29.6, 28.1, 23.4, 18.3, 18.1, 16.6, 9.3. IR (film) 3348 (OH), 1732 (C=O), 1650 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{23}H_{34}O_6Na$: 429.2253 (M^++Na). Found: 429.2265.

Compound **19**: Mp 221–223 °C (MeOH). $[\alpha]_D$ −45.6 (*c* 0.15, MeOH). ^{1}H NMR (400 MHz, 300 K, $CDCl_3$) δ 6.25 (1H, s), 5.57 (1H, s), 5.37 (1H, s), 5.11 (1H, s), 5.04 (1H, ddd, 3.8, 3.8, 3.8), 4.40 (1H, ddd, 12.0, 4.2, 4.2), 3.91 (1H, d, 11.0), 3.67 (1H, d, 11.0), 3.19 (1H, d, 3.0), 2.86 (1H, d, 4.2), 2.44 (4H, m), 1.88 (1H, m), 1.78 (1H, ddd, 12.1, 12.1, 12.1), 1.73–1.70 (2H, m), 1.60 (2H, m), 1.49 (1H, m), 1.43–1.31 (3H, m), 1.25 (1H, m), 1.21 (3H, s), 1.18 (3H, t, 7.4), 1.17 (3H, t, 7.6), 0.89 (3H, s), 0.68 (1H, m). ^{13}C NMR (100 MHz, 300 K, $CDCl_3$) δ 207.2, 174.6, 173.4, 143.9, 120.6, 74.7, 73.3, 72.0, 70.6, 60.8, 54.5, 50.3, 46.7, 38.8, 38.6, 36.5, 35.2, 28.0, 27.9, 27.7, 23.0, 17.6, 17.4, 16.3, 9.3, 9.0. IR (film) 3381 (OH), 1738, 1723, 1709 (C=O), 1644 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{26}H_{38}O_7Na$: 485.2515 (M^++Na). Found: 485.2521.

4.3.16. Preparation of 20 and 21 from 2

A mixture of **2** (0.036 g, 0.092 mmol), butyric anhydride (0.044 g, 0.046 mL, 0.28 mmol), dry Et_3N (0.037 g, 0.051 mL, 0.37 mmol), a catalytic amount of DMAP, and dry CH_2Cl_2 (1 mL) was stirred at rt for 5 h. The reaction mixture was diluted with AcOEt (20 mL) and the organic layer was separated. The aqueous layer was washed with AcOEt (10 mL × 3) and the combined organic layer and washings was washed with 5%w/v HCl (20 mL × 2), satd $NaHCO_3$ (20 mL × 2), and satd NaCl (20 mL × 2), successively, dried over $MgSO_4$, and filtered. The filtrate was subjected to evaporation in vacuo to give an oily residue, which, by SiO_2-MPLC (hexane/AcOEt = 4:1), gave a crude diacyl compound (0.035 g). A mixture of the crude diacyl compound (0.025 g, 0.048 mmol), 1 N HCl (1.0 mL), and MeOH (5.0 mL) was stirred at rt for 96 h. After dilution with H_2O (30 mL), the mixture was extracted with AcOEt (10 mL × 3), and the extract was washed with satd $NaHCO_3$ (20 mL × 2) and NaCl (20 mL × 2), successively, dried over $MgSO_4$, and filtered. The solvent was evaporated off in vacuo to give a colorless solid, which was subjected to HPLC (80% MeOH, v/v) to give **20** (0.006 g, 22%, two steps overall yield) as a colorless solid and **21** (0.0087 g, 27%, two steps overall yield) as a colorless solid.

Compound **20**: Mp 208–210 °C (MeOH). $[\alpha]_D$ −47.7 (*c* 0.12, MeOH). ^{1}H NMR (500 MHz, 300 K, pyridine-d_5) δ 8.27 (1H, d, 4.9), 7.44 (1H, s), 6.34 (1H, s), 6.19 (1H, br m), 5.55 (1H, s), 5.49 (1H, s), 5.26 (1H, dd, 4.8, 4.8), 5.05 (1H, ddd, 12.1, 4.4, 4.4), 3.69 (1H, dd, 10.4, 3.9), 3.57 (1H, d, 3.3), 3.34 (1H, dd, 10.4, 3.9), 2.48–2.42 (2H, m), 2.08 (1H, ddd, 12.3, 12.3, 12.3), 1.85–1.61 (8H, m), 1.54 (1H, br d), 1.44–1.36 (3H, m), 1.35 (3H, s), 0.96 (3H, t, 7.4), 0.92 (3H, s), 0.63 (1H, m). ^{13}C NMR (125 MHz, 300 K, pyridine-d_5) δ 207.7, 172.6, 146.2, 118.8, 74.32, 74.26, 71.3, 71.2, 61.1, 55.4, 51.4, 46.2, 39.4, 38.8, 38.0, 36.6, 35.5, 29.6, 23.4, 18.7, 18.3, 18.1, 16.6, 13.8. IR (film) 3348 (OH), 1732 (C=O), 1649 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{24}H_{36}O_6Na$: 443.2410 (M^++Na). Found: 443.2394.

Compound **21**: Mp 163–165 °C (MeOH). $[\alpha]_D$ −36.9 (*c* 0.17, MeOH). ^{1}H NMR (500 MHz, 300 K, pyridine-d_5) δ 8.40 (1H, d, 5.3), 7.38 (1H, s), 6.35 (1H, s), 5.51 (1H, br m), 5.50 (1H, br s), 5.26 (1H, dd, 3.7, 3.7), 4.86 (1H, ddd, 12.1, 4.8, 4.8), 4.11 (1H, d, 11.0), 3.75 (1H, d, 11.0), 3.56 (1H, d, 3.4), 2.43 (2H, m), 2.22 (2H, m), 2.18 (1H, m), 2.04 (1H, ddd, 12.4, 12.4, 12.4), 1.79–1.67 (3H, m), 1.61–1.55 (4H, m), 1.52 (1H, m), 1.43–1.32 (5H, m), 1.29 (3H, s), 0.96 (3H, t, 7.4), 0.88 (3H, s), 0.85 (3H, t, 7.4), 0.59 (1H, ddd, 12.6, 12.6, 3.2). ^{13}C NMR (125 MHz, 300 K, pyridine-d_5) δ 207.5, 173.2, 172.6, 146.0, 119.0, 74.1, 74.0, 72.20, 71.18, 61.0, 55.2, 51.3, 46.9, 39.1, 38.8, 36.69, 36.63, 36.2, 35.7, 29.7, 23.4, 18.8, 18.7, 17.9, 17.6, 16.5, 13.79, 13.78. IR (film) 3376 (OH), 1733 (C=O), 1650 (C=C) cm^{-1}. HRMS (ESI): Calcd for $C_{28}H_{42}O_7Na$: 513.2828 (M^++Na). Found: 513.2802.

4.4. Biological activity assay

4.4.1. Inhibitory effect on LPS-induced NO production by macrophages

RAW267 macrophages (5×10^5/200 μl/well, RIKEN cell bank, Tsukuba) were placed in each well of a 96 well culture plate and incubated with recombinant murine IFN-γ at 2 ng/ml for 24 h at 37 °C. After incubation, the solution was removed and the cells were treated with the test compound by adding a compound solution (0.4, 2.0, and 10.0 μg/mL) in RPMI1640 containing 10% fetal bovine serum to each well (100 μl/well) and leaving for 30 min at 37 °C. After the incubation, lipopolysaccharide (LPS, Sigma) solution 200 ng/ml in RPMI1640 containing 10% FBS (100 μl/well) at 37 °C for 24 h, was added to each well, the cell-free culture supernatant was collected for the estimation of the amount of the released NO by the nitrite content assay. Briefly, the cell-free supernatant (50 μl) was reacted with the same volume of Greiss reagent (1% sulfanilamide, 0.1% naphthylethylene diaminedihydrochloride and 2.5% phosphoric acid) for 10 min at rt as described[14] and its optical density was measured at 540 nm. The nitrite content was determined by the comparison with a sodium nitrite standard curve (0–100 μM).

4.4.2. Cell viability test on RAW267 macrophages

The RAW264 cells left on the cell well surface after the NO production assay were incubated with WST reagent (Dojin) for 2 h at 37 °C. After the incubation, the absorbance at 450 nm of the culture supernatant was measured by microplate reader (Corona MTP 450). The higher OD450 implies higher cell viability.

4.4.3. Luciferase-assisted assay of inhibitory effect on NF-κB activation

293T cells in DMEM containing 10% FBS were placed in each well of a 96-well plate (100 μl, 2×10^4 cells/well) and incubated for 20 h at 37 °C under 5% CO_2 atmosphere. Transfection was performed by adding to each well a transfection mixture consisting of Lipofectamine LTX (Invitrogen, 0.25 μl/well), PLUS Reagent (Invitrogen, 0.05 μl/well) and Plasmid DNA mixture (p3x-FLAG-CMV 9/TLR4, pBud-CE4/MD-2, pGL4.32[luc2P/NF-κB-RE/Hygro] Vector, and pGL4.74[hRluc/TK], 82 ng/well) (10 μl/well) and incubating the plate at 37 °C for 20 h. After transfection, the cells were treated with the test compound solution (0.32, 0.8, 4.0, and 20.0 μM) in RPMI1640 containing 10% fetal bovine serum (50 μl/well) for 30 min at 37 °C, then lipopolysaccharide solution 400 ng/ml (Sigma) (50 μl/well) was added to each well, and the plate was incubated at 37 °C for 6 h. After removing the supernatant solution, the cells were lysed with Passive Lysis Buffer (Promega)(20 μl /well) at room temperature and the lysate was assayed by the Dual-Luciferase Reporter Assay System (Promega). The luciferase assay reagent (50 μl) was added to the lysate (10 μL), and 2 s later, the chemiluminescence measurement of the mixture was done for 10 s by using the Microplate Luminometer (Berthold Technologies GmbH, KG, Bad Wildbad, Germany).

4.4.4. 293T cell viability test

The 293T cell lysate prepared for the NF-κB activation assay described above was used for the cell viability test by using the Microplate Luminometer (Berthold Technologies GmbH, KG, Bad Wildbad, Germany). Since the renilla luciferase expression is controlled by the constitutive active thymidine kinase promoter in pGL4.74[hRluc/TK] construct, the enzyme activity reflects the cell viability. Thus the chemiluminescence generated by renilla luciferase and the 2nd specific substrate of the Dual-Luciferase Reporter Assay System (Promega) were measured for 10 s. Viable cell ratio was calculated from the chemiluminescense intensity of the control cells lysate treated only with the vehicle and LPS.

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